From THE DEPARTMENT OF NEUROSCIENCE
Karolinska Institutet, Stockholm, Sweden

BRAIN DEVELOPMENT IN A DISH

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Stockholm 2020

Cover: “Tying together the brain and a dish” by Robin Johan Pronk
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Published by Karolinska Institutet.
Printed by Universitetsservice US-AB 2020

ISBN 978-91-7831-770-7

Brain Development In A Dish

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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Public defense: Friday 27th of March 2020
Lecture hall: Biomedicum 1, 09:30
Solnavägen 9

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"Essentially, all models are wrong, but some are useful"

George E.P. Box

ABSTRACT

This thesis shows the path I took in the quest for modeling the brain, as the title says, in a dish. The introduction of induced pluripotent stem cells (iPSCs) as means to research biological processes has opened up whole new fields of study and unprecedented possibilities of generating human cells *in vitro*. iPSCs have due to their pluripotent state the theoretical ability to be an unlimited source of cells, capable to generate any cell of the human body. They can be derived from somatic tissue and therefore used to generate disease specific cells. In **paper I**, we show the generation of disease specific neural stem cells from lissencephaly patients carrying a mutation in doublecortin (*DCX)*. Lissencephaly is a disorder that affects cell migration, a phenotype we could replicate in our model. Furthermore, we show a defect in proper neurite outgrowth that we could rescue through the SLIT/ROBO pathway, and a prolonged proliferation. Together, showing the feasibility of using iPSC derived neural stem cells to model human neurodevelopmental disorders such as lissencephaly. In **paper II** we explored the role of p53 in neurodevelopment using both iPSC derived neuroepithelial stem cells (NES) and 3D brain organoids. Here we used lentiviral knockdown of tumor protein (TP53) in both the NES and iPSCs to follow neural development. We show the importance of p53 in maintaining genomic stability of NES cells and the involvement in maintaining the metabolic balance, resulting in lower expression of oxidative phosphorylation (OXPHOS) genes, shifting the cells to a more glycolytic state. Further differentiation into neurons showed an increased pace of differentiating. When placing p53 in the context of brain organoids, we show the reduction of $TBR2^+$ intermediate progenitor cells (IPCs) and $TBR1^+$ neurons. Analyzing metabolic gene profile also revealed the downregulation of OXPHOS related genes, indicating the regulation by p53 of the metabolism in brain organoids. In the **manuscript**, we explored a more metrological aspect for modeling neurodevelopmental diseases. By generating brain organoids and evaluating the neuronal activity we show the feasibility for future drug screening. Furthermore, we could perform an *in vitro* transplantation of NES cells and show their differentiation, making a small step towards an alternative for *in vivo* transplantation.

In summary, this thesis shows some of the potential of *in vitro* brain development and the applications these brain models can be used for.

NEDERLANDSE SAMENVATTING

Dit proefschrift toont het pad dat ik heb gevolgd in de zoektocht naar het nabootsen van de hersenen, zoals de titel al luid, in een schaal. De introductie van geïnduceerde pluripotente stamcellen (iPSC's) als middel om biologische processen te onderzoeken, heeft volstrekt nieuwe onderzoeksgebieden en ongekende mogelijkheden voor het genereren van menselijke cellen *in vitro* geopend. iPSC's hebben vanwege hun pluripotentie het theoretische vermogen om een onbeperkte bron van cellen te zijn, in staat om elke cel van het menselijk lichaam te genereren. Ze kunnen worden gemaakt van somatisch weefsel en daarom worden gebruikt om ziekte specifieke cellen te genereren. In **artikel I** laten we de generatie van ziekte specifieke neurale stamcellen zien van lissencefalie patiënten met een mutatie in het doublecortin (DCX) gen. Lissencefalie is een aandoening die celmigratie beïnvloedt, een fenotype dat we in ons model kunnen repliceren. Bovendien tonen we een defect in de uitgroei van neurieten aan die we via het SLIT / ROBO-pad kunnen redden. Samengenomen tonen we de haalbaarheid van het gebruik van van iPSC afgeleide neurale stamcellen om menselijke neurologische ontwikkelingsstoornissen zoals lissencefalie te modelleren. In **artikel II** hebben we de rol van p53 in de neurologische ontwikkeling onderzocht met behulp van iPSC-afgeleide neuroepitheliale stamcellen (NES) en 3D-hersenorganoïden. Hier hebben we lentivirale knockdown van tumor-eiwit (TP53) gebruikt in zowel de NES als iPSCs om de neurale ontwikkeling te volgen. We tonen het belang van p53 aan bij het handhaven van de genomische stabiliteit van NES-cellen en de betrokkenheid bij het handhaven van de metabolische balans, resulterend in een lagere expressie van oxidatieve fosforylatie (OXPHOS) genen, waardoor de cellen meer naar een meer glycolytische toestand worden verplaatst. Verdere differentiatie in neuronen toonde een verhoogd tempo van differentiatie. Wanneer we p53 in de context van hersenorganoïden plaatsen, tonen we de reductie van $TBR2^+$ intermediaire voorlopercellen (IPC's) en $TBR1^+$ neuronen aan. Analyse van het metabole genprofiel onthulde ook de reductie van OXPHOS-gerelateerde genen expressie, hetgeen duidt op de regulatie door van het metabolisme in hersenorganoïden door p53. In het **manuscript** hebben we een meer metrologisch aspect onderzocht voor het modelleren van neurologische aandoeningen. Door hersenorganoïden te genereren en de neuronale activiteit te evalueren, toonden we de haalbaarheid aan voor toekomstige screening van geneesmiddelen. Verder slaagden we in het uitvoeren van een *in vitro* transplantatie van NES-cellen en konden we tevens hun differentiatie laten zien, waarmee we een stap zetten naar een alternatief voor in vivo transplantatie.

Samenvattend, toont dit proefschrift een deel van het potentieel van *in vitro* hersenontwikkeling aan en de toepassingen waarvoor deze hersenmodellen kunnen worden gebruikt.

SCIENTIFIC PAPERS INCLUDED IN THE THESIS

I. An *in vitro* model of lissencephaly: expanding the role of DCX during neurogenesis

M Shahsavani, **RJ Pronk**, R Falk, M Lam, M Moslem, SB Linker, J Salma, K Day, J Schuster, B-M Anderlid, N Dahl, FH Gage, and A Falk

Molecular Psychiatry, doi:10.1038/mp.2017.175

II. p53 controls genomic stability and temporal differentiation of human neural stem cells and affects neural organization in human brain organoids

Ana Marin Navarro[#], **Robin Johan Pronk[#]**, Astrid Tjitske van der Geest, Ganna Oliynyk, Ann Nordgren, Marie Arsenian-Henriksson, Anna Falk, and Margareta Wilhelm

Cell Death and Disease, doi: 10.1038/s41419-019-2208-7

[#]Authors contributed equally

III. Generation of a 2D and 3D System to Understand Neuropsychiatric Disorders

Pronk R.J., Hauger P.C., Marin Navarro A, Wilhelm M, and Falk A

Manuscript in preparation

SCIENTIFIC PAPER NOT INCLUDED IN THE THESIS

IV. Single cell analysis of autism patient with bi-allelic NRXN1-alpha deletion reveals skewed fate choice in neural progenitors and impaired neuronal functionality

Lam, M, Moslem, M[#], Bryois, J[#], **Pronk, R. J**, Uhlin, E, Ellstrom, I. D, Laan, L, Olive, J, Morse, R, Ronnholm, H, Louhivuori, L, Korol, S. V, Dahl, N, Uhlen, P, Anderlid, B. M, Kele, M, Sullivan, P. F, and Falk, A.

Experimental Cell Research: doi: 10.1016/j.yexcr.2019.06.014

[#]Authors contributed equally

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LIST OF ABBREVIATIONS

2D	Two-Dimensional
3D	Three-Dimensional
ANLSH	Astrocyte to Neuron Lactate Shuttle
AP	Action Potential
ARX	Aristaless-Related homeobox protein
ASD	Autism Spectrum Disorder
ATP	Adenosine Triphosphate
BLBP/FABP7	Brain Lipid Binding Protein/ Fatty Acid Binding Protein 7
BMP	Bone Morphogenetic Protein
Ccas3	Cleaved Caspase-3
CNS	Central Nervous System
DCX	Doublecortin
DECR1	2,4-Dienoyl-CoA Reductase 1
DNA	Deoxyribonucleic Acid
E13,5	Embryonic day 13,5
EBs	Embryoid Bodies
EdU	5-Ethynyl-2' -Deoxyuridine
EEG	Electroencephalogram
ESC	Embryonic Stem Cell
FGF	Fibroblast Growth Factor
G1-phase	Gap1-phase
GFAP	Glial Fibrillary Acidic Protein
GLAST	Glutamate Aspartate Transporter 1
GWAS	Genome Wide Association Study
HES5	Hairy Enhancer of Split 5 / Hes Family BHLH Transcription Factor 5
HK2	Hexokinase 2
IKNM	Inter Kinetic Nuclear Movement
IPC	Intermediate Progenitor Cell

iPSCs	Induced Pluripotent Stem Cell
KD	Knock Down
KI67	Marker of Proliferation KI-67
KO	Knock Out
MACS	Magnetic Associated Cell Sort
MEA	Multi Electrode Array
NES	Neuroepithelial Stem Cell
NPC	Neural Progenitor Cell
NTD	Neural Tube Closure Defects
oRG	Outer Radial Glia
OSKM	Oct4, Sox2, Klf4, c-Myc, “Yamanaka factors”
oSVZ	Outer Subventricular Zone
OXPHOS	Oxidative Phosphorylation
PAFAH1B1	Platelet-Activating Factor Acetyl Hydrolase isoform 1B
PSA-NCAM	Polysialylated-Neural Cell Adhesion Molecule
PSC	Pluripotent Stem Cell
RELN	Reelin
RG	Radial Glia
ROBO1-2	Roundabout Guidance Receptor 1
ROCK	Rho Associated Coiled-Coil Containing Protein Kinase 1
S-phase	Synthesis-Phase
SBH	Subcortical Band Heterotopia
SFEBq	Serum Free culture of Embryoid Body like aggregates Quick
shRNA	Short Hairpin RNA
SLIT3	Slit Guidance Ligand 3
SOX2	Sex determining region Y-Box2
SVZ	Sub Ventricular Zone
SZ	Schizophrenia
TCA	Tricarboxylic Acid Cycle

TP53	Tumor Protein 53
tRG	Truncated Radial Glia
TUBA1A	Tubulin Alpha 1A
TUJ1	Class III b-Tubulin
RB	Retinoblastoma
VLDLR	Very Low Density Lipoprotein Receptor
vRG	Ventricular Radial Glia
WT	Wild Type

1 GENERAL INTRODUCTION

The 20th century was the starting point for "Modern Neuroscience". Most famously, Camillo Golgi (July 7th, 1843 – January 21st, 1926) and Santiago Ramón y Cajal (May 1st 1852 – October 17th 1934). The two inspiring scientists had opposing views on how neurons formed a network, but were both awarded the Nobel Prize in Physiology or Medicine (1906). Golgi developed the "Black Reaction" (nowadays known as the Golgi stain) [1], in which only a few percent of neurons are labeled entirely black. To date, it is not known why only a subset of cells are stained or, why particularly those cells are stained [2]. Panel 1 in Figure 1 shows a drawing made by Golgi, displaying cell types in the human cerebral cortex [1,3]. Panel 2 shows a photomicrograph of a pyramidal neuron in the cerebral cortex. One can appreciate the similarities between the drawing and the photograph, made over 100 years later.

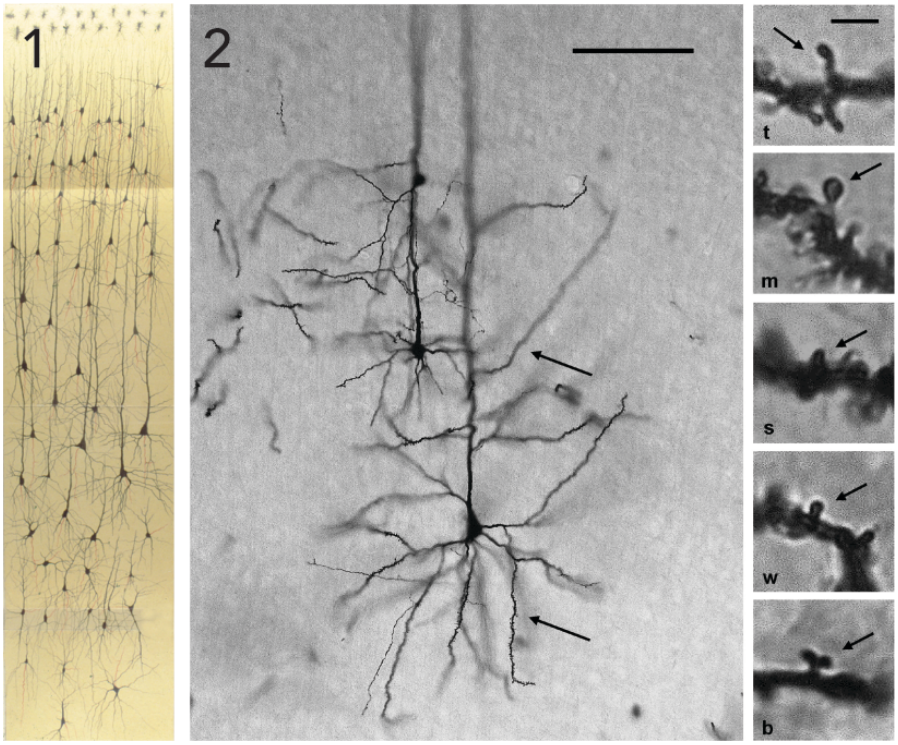

Figure 1: ***Panel 1****: Golgi's drawing of the cell types of the human cerebral cortex. Golgi divided the cortex into three laminae: a superficial layer with small pyramidal cells; a middle layer with larger pyramidal cells; and a deep layer with fusiform cells. Reproduced with permission from the publisher from Glickstein 2006, original drawing from Golgi 1885.*
Panel 2*: Left panel: Photomicrograph of a fifth-layer pyramidal neuron in the motor cortex. Arrows show the apical and basal dendritic branchlets from where the spines were counted. Scale bar = 150 µm. Right panel: Photomicrographs of the thin (t), mushroom (m), stubby (s), wide (w), and branched (b) spines (arrows); as those counted in this study. Scale bar = 2 µm. Reproduced with permission from the publisher from Vazquez-Hernandez et al., 2017.*

It was with the staining technique from Golgi that Cajal contributed to the growing theory that neurons were not physically joined together (The Neuron Theory) and thus did not form a continuous network (The Reticular Theory). Therefore, Cajal established further the concept that neurons are individual units, part of a bigger network. The irony of it all is reflected in Cajal's comment of shared Nobel Prize:" *What a cruel irony of fate of pair, like Siamese twins united by the shoulders, scientific adversaries of such contrasting character!*". Golgi and Cajal had similar methods, but came to a fundamental different hypotheses in their understanding of the nervous system. In the same year as Golgi and Cajal were awarded the Nobel Prize, Sherrington laid the groundwork of an information transfer system between neurons (first published in 1906 [4]). It was not until 50 years later, with the use of the electron microscope, that the unequivocal existence of the synapse, and thus the view of neurons as individual units was published [5,6].

With that in mind, I like to reflect that, the methods of today dictate the theories, but the future holds the answer.

1.1 BRAIN DEVELOPMENT

There are countless cell types in the brain, each with their own regional specification, generated in a timely manner (**Neurogenesis** followed by **Gliogenesis**) and guided to their place by a plethora of signaling factors and pathways. Once these cells find their way (**Migration**), climbing under, over, and sideways along other cells, they arrive at their destination. Presumably the environment informing them, this is home, settle in, it was a long journey. After they are settled, the social butterflies they are, they want to meet and connect with many more similarly minded cells. With a process that includes more and more dendrites and axonal projections to travel to potential new friends, guided by factors to attract and repulse (**Neurite outgrowth**). Every time they find a fellow likeminded cell, they will connect, forming a synapse allowing them to communicate (**Neural signaling**). When you keep in touch with your friends, this connection grows in strength over time, and dwindles when neglected. Therefore, constantly shaping the brain and its connections as it seems fit through development and continuing well into the 3th decade of life.

Now in this brief overview of brain development there are several topics in relation to this thesis that I will discuss in the following chapters

1.2 FROM NEURAL STEM CELLS TO THE BRAIN

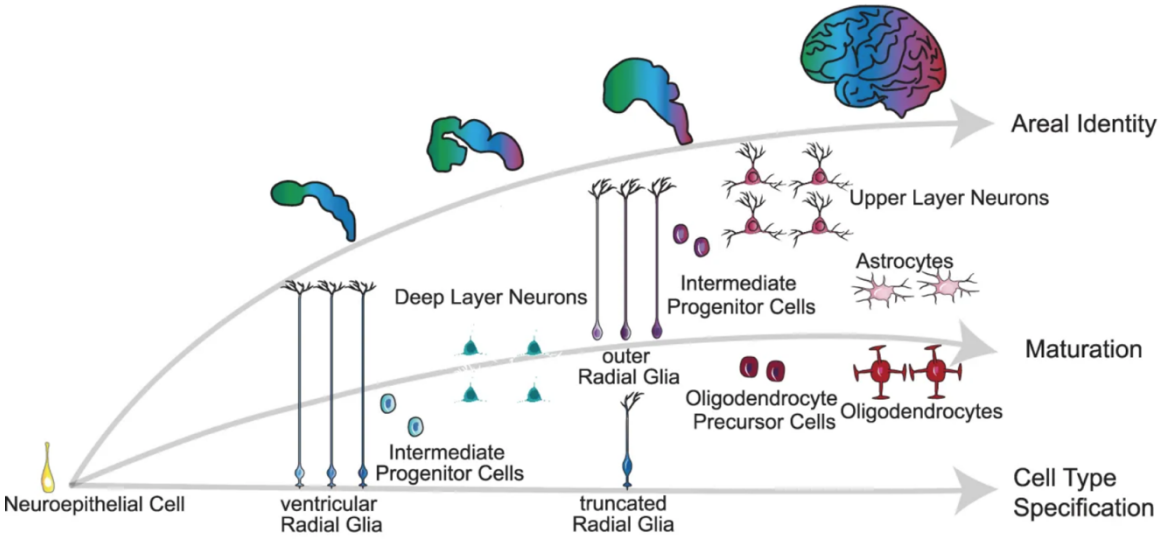

Figure 2: General overview of human brain development, adapted with permission from the publisher, Bhaduri et al., 2020

1.2.1 Neuroepithelial stem cells

Neuroepithelial stem cells (NES) form the foundation on which the brain is built. They show a polarized apical-basal organization, with adherence as well as tight junctions at the apical end of the cell maintaining polarity and organization [7-11] (The side lining the ventricle). This layer of cells looks pseudostratified, due to the interkinetic nuclear movement (IKNM) of the nuclei [7,8,12-14]. When observed in sections, the cells are still one layer, but have their nuclei on different levels. The IKNM corresponds to a specific phase in cell cycle [15,16]. After advancing through the cell cycle, NES cells divide either symmetrically, or a-symmetrically. This leads to first the lateral expansion of the neo-cortex by symmetrical division [17], and second to an a-symmetrical division that yields a daughter NES cell and the first group of neurons [18,19]. Besides symmetrical division and generation of more NES cells, these stem cells later turn into radial glia cells.

1.2.2 Radial Glia

Radial Glia (RG) cells, first identified as scaffold for migrating neurons [20,21] share many features with NES cells, and were therefore difficult to distinguish. RG as well as NES cells, contact both the ventricular and pial surface of the developing brain, and exhibit IKNM [22]. However, while in the NES cells this movement is across the entire cell, RG nuclei only migrate across the span of the ventricular zone. Overlap in the expression of NESTIN [23,24], marks them as well as stem cells, and the extension of a cilium contacting the cerebrospinal fluid in both NES cells and RG cells [25,26], further established their similarities. The transition from NES cells to RG cells comes with astroglial features. Glial fibrillary acidic protein (GFAP) is expressed in astrocytes and primate radial glia [20], where the delta isoform of GFAP is specific to the sub-ventricular zone (SVZ) in humans [27] but not in mice [28]. Brain lipid-binding protein (BLBP) [29,30] and glutamate aspartate transporter (GLAST) [31] are more specific to radial glia in development, while in later developmental stages these markers become restricted to astrocytes. Besides just acting as a scaffold as originally thought [20,21], RG cells serve as main neurogenic centers in the developing cortex [32-34]. The potential of RG cells is thus far described as multi-potent. At early stages, RG cell divide to expand their progenitor pool, while at later stages a-symmetric divisions generating a RG cell and an intermediate progenitor (IP) cell become more standard [35,36]. IPs line an area adjacent to the ventricular zone in the developing cortex and are marked by SVET1 and TBR2 expression [37,38]. Later in human development, the progenitor pool expands with radial glia types such as, ventricular RG (vRG), truncated RG (tRG), [39] and outer RG (oRG) [40] populating the inner- and outer-subventricular zone (iSVZ and oSVZ, respectively). With the finding

that a singular oRG cells can produce hundreds of deep and upper cortical layer neurons, it is thought that these cells are significant contributors to the enlarged oSVZ seen in humans [41]. The generated neurons have to migrate away from this niche to their respective place in the cortex. During neurogenesis in the cortex, the layers are generated in an "inside out" manner [42,43], meaning the newest neurons have to migrate past existing ones.

1.2.3 Migration

During the early neurogenic period, NES cells divide a-symmetrically, giving rise to an equally potent daughter stem cell and a neuron [44,45]. For this neuron to reach its destination it will migrate from the apical surface to the basal surface. As observed in the fetal monkey cortex, migrating neurons are in close proximity to RG fibers, which act as support and guidance for migrating neurons [46]. By live cell imaging it can be observed that newborn cortical neurons migrate by forming a leading edge that is protruded from the neuron, followed by soma translocation [47]. It is evident that the movement of cells in general requires plenty of structural changes to the cell's cytoskeleton. A major component of the cytoskeleton are microtubules which regulate and facilitate polarity and direction of migrating neurons [48,49], as well as coupling of the leading edge to the movement of the nucleus [50]. Microtubules themselves consist of alpha- and beta-subunits connected to form long tubes, stabilized by microtubule associated proteins, one of which is doublecortin (DCX). This protein is stabilizing microtubules in migrating and differentiating neurons as their growth cones are pathfinding and the cell is dragged along its chosen path [51,52].

1.2.3.1 Lissencephaly – A Neuronal Migration Disorder

When newborn neurons fail to reach their respective destination, they settle randomly, resulting in a disorder called lissencephaly [53,54] ("smooth brain", from the Greek "lissos", smooth and, "enkephalos", brain). Normally neatly organized in six separate layers, the cortex of patients suffering from lissencephaly often present with only fewer distinct layers and with diminished to absent gyrification. Lissencephaly can be caused by mutations in several genes such as: *PAFAH1B1* (*LIS1*), *ARX*, *RELN*, *VLDLR*, *TUBA1A*, and *DCX* (as listed previously [55] and shown in Figure 3).

Of these, *PAFAH1B1* (*LIS1*) and *DCX* mutations account for roughly 76% of classical lissencephaly cases [56]. Described as a classical neural migration disorder [57], DCX dependent lissencephaly is an X-linked disorder [58]. DCX is expressed in migrating neurons of the human brain [59] and regulates the stability and organization of microtubules [52,59,60]. In females, due to the random X-inactivation in development, two populations of migration neurons are formed. One population with WT DCX form and function, and another population with less or non-functional DCX, leading to subcortical band heterotopia (SBH) [61]. Therefore, the phenotype seen in males is more severe with substantially diminished or a lack of gyrification. Varying mutations in the *DCX* gene are associated with a heterogeneity of phenotypes [62,63]. The *DCX* gene encodes for a brain specific, 360 amino acids long and a predicted 40 kDa protein [57,58]. Interestingly, the phenotypes in human are not replicated in a knockout (KO) mouse model [64]. The authors show the lamination of the hippocampus is disrupted, however the layering of the cortex is unaltered. Later research hinted towards a compensatory mechanism in mice, that evidently is not-existent or insufficient in humans [65,66].

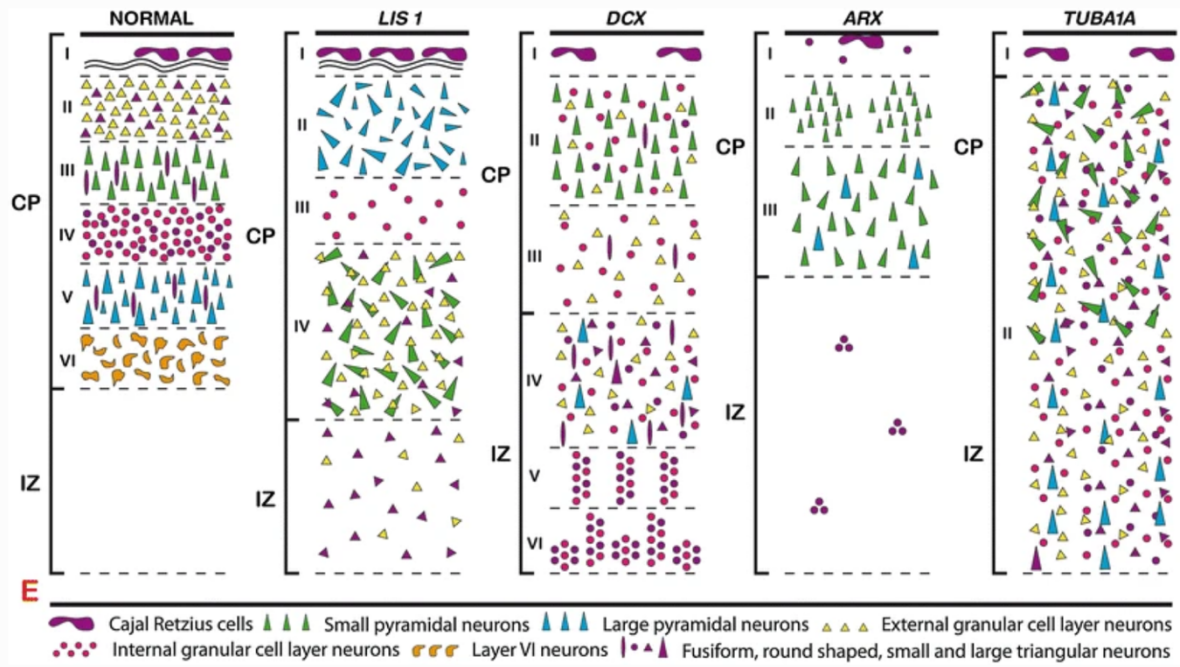

Figure 3: Schematic representation of the localization of different neuronal populations in the cerebral mantle of normal and lissencephalic brains, highlighting the differences of cortical lamination defects. CP cortical plate, IZ intermediate zone, E ependyma. Reproduced from Friocourt et al., 2010 [67]. With permission from the journal

1.2.4 Neurite outgrowth

Whether it is some form of extension that grows out of a migrating neuron at its leading edge, or the extensions that later form the axons and dendrites in the brain, they rely heavily on microtubules and their stabilization [53,68]. For the cell to decide the direction it needs to grow in, it needs some guidance. Chemo attractants and chemo repellents, like the name suggests, attract and repel neurons and neurites that are making their way through the brain. Several navigational mechanisms have been described [69], of which many have been found to be an attractant in one scenario and a repellent in another [70,71]. Factors such as differential receptor activation [72] and different levels of intracellular second messengers [73] can aid in the switch from an attractant to repellant and vice versa. One of the navigational mechanism migration neurons and neurites use is the SLIT/ROBO pathway [74,75]. Newly born neurons use this pathway to migrate through the glial meshwork [76] and a knockdown (KD) of SLIT 3 and added ROBO1/2 protein promotes neurite outgrowth in lissencephaly patients cells where impaired neurite outgrowth is observed [77].

1.3 P53 IN BRIEF

The transcription factor p53 is most famous for its role as tumor suppressor, since the gene is mutated in approximately 50% of all human cancers [78]. There is a strong positive correlation between larger animals and their lifespan [79]. Interestingly, TP53 copy number expansion is associated with an increased body size during evolution [79]. The 20 copies of TP53 retrogenes that elephants carry and their enhanced DNA damage response [79,80] show a remarkable, albeit extreme difference in p53 within mammals. p53, famously known as guardian of the genome [81], made its appearance in literature in 1979 [82,83]. First thought to be an oncogene, undoubtedly because of its mutated form being present in so many cancers. Later work showed the wildtype (WT) variant to be one of the most important tumor suppressors known to date [84]. Over the years a plethora of p53 functions have come to light. As summarized previously [85] (Figure 4), involvement of p53 has been shown in, but not limited to: Apoptosis, Autophagy, Cell cycle, Differentiation, DNA damage and response, Inflammation, Metabolism, Proliferation, Reactive oxygen species and, Stress response.

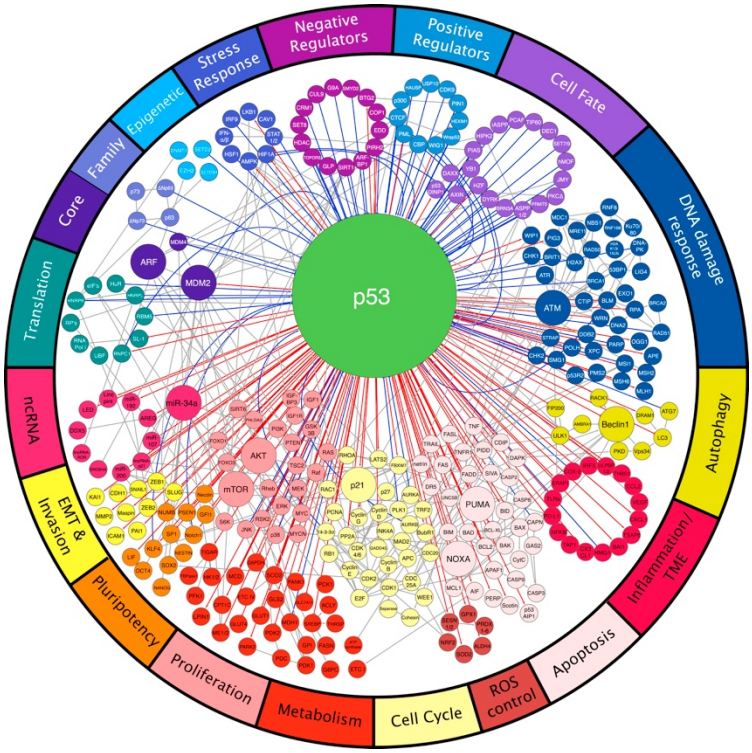

Figure 4: The p53 network. Kastenhuber and Lowe, 2017, reproduced with permission from the publisher.

1.3.1 P53 in development

The first indication for the role of p53 in development were surprisingly mild, considering the array of functions it is involved in. The *p53* KO mouse is reported to "develop normally", but is vulnerable to tumor development [86]. In humans, it is known that a mono-allelic mutation of *TP53* leads to Li-Fraumeni syndrome. Here, affected individuals carry heterozygous germline mutations in the *TP53* gene. These individuals usually develop cancers during their lifetime [87,88] and even with the exclusion of more sex-specific cancers (breast, ovarian, and prostate cancer), the incidence is still higher in females than in males [89,90]. The heavier burden in females is especially present when observing *p53* KO mice. Not only are female mice underrepresented [91], a subset of female mice develop exencephaly following neural tube closure defects (NTDs), whereas $p53^{-/-}$ males do not exhibit NTDs [91,92]. The penetrance for NTDs in females varies between 17-60% depending on genetic background and the conducted study [91,92]. Only recently, it has been shown that the NTDs are due to the binding of p53 to the *X-chromosome inactivation center* (*XIC*) and thereby promoting the stochastical inactivation of one of the female X-chromosomes [93]. The authors show, that in absence of p53 *Xist* expression is reduced, which leads to a subsequent diminished number of *Xist* clouds in a subset of cells, suggesting the direct activation of *XIC* genes by p53. Without p53 some cells are not able to repress *XIC* genes and therefore cause NTDs.

1.4 METABOLISM

Talking about the metabolism of a cell, we mainly refer to two energy producing pathways: Glycolysis and Oxidative phosphorylation (OXPHOS). It has been shown that during the development from a neural stem cell to a neuron, the progenitor cell relies more on glycolysis, whereas the neurons rely on OXPHOS [94,95]. The developing embryo is almost exclusively glycolytic throughout the neural tube, neural crest, and brain vesicle development [96]. In terms of net energy production, glycolysis is relatively inefficient with two ATPs (anaerobic glycolysis) or four ATPs generated (aerobic glycolysis) versus OXPHOS with 36 ATPs. OXPHOS and glycolysis are not mutually exclusive processes, a particular cell can possibly utilize both pathways. Interestingly, recent findings point towards a metabolic collaboration between neurons, resident astrocytes, and oligodendrocytes, named the astrocyte-oligodendrocyte-neuron lactate shuttle (AONLSH) [97,98]. One of the key components is, as the name suggests, the use of glycolysis (glucose is eventually metabolized to lactate via pyruvate) by astrocytes and oligodendrocytes as a source for interstitial lactate. This lactate is taken up by the neurons and used in the OXPHOS pathway, where lactate is converted to pyruvate, which is essential for the production of Acetyl-coA by the TCA cycle, which in turn produces NADH

for the electron transport chain and the OXPHOS generation of ATPs [99,100]. The question is, to what extend the neurons can self-support their energy needs in the absence of astrocytes. Gliogenesis generally occurs only after all neuronal layers are formed, thus there is a significant period of astrocyte absence in early brain development. One of the hallmarks of differentiating neurons is the requirement of making a glycolysis to OXPHOS switch. Neural stem cells are mainly glycolytic [95] and require a connection between aerobic glycolysis and OXPHOS for differentiation [101]. Downregulation of glycolysis genes is essential for neuronal differentiation [95] and genetic inhibition of mitochondria (OXPHOS) impairs neurogenesis *in vivo* [102] thus indicating the need for balance and versatility of a proper metabolic energy profile.

1.5 UNDERSTANDING NEUROPSYCHIATRIC DISORDERS

Neuropsychiatric disorders might very well be one of the most difficult conditions to understand, considering the relative limited knowledge we have about the brain. In cases of autism spectrum disorder (ASD) and schizophrenia (SZ) some phenotypes like communication difficulties are shared with common genetic overlap [103,104] thus affecting the "Social brain" [105], that can be associated to prefrontal cortical development [106]. As shown by valiant efforts of many laboratories, the complexity of these diseases is enormous. Genome Wide Association Studies (GWAS) have identified many different mutation hotspots and copy number variations (CNVs) [103,107,108], some CNVs with significant overlap between ASD and SZ [104]. Efforts are towards recapitulating what is called the DSM-V classification of in this case ASD and SZ. Diagnosis is made through thorough clinical evaluation in the case of ASD: Deficits in social-emotional reciprocity, nonverbal communication, developing and maintaining relationships and more. And for SZ: "delusions, hallucinations, diminished emotional expression and others". For full DSM classification and explanation, see DSM-V [109]. Research is constantly developing and the more we know about the brain and its functions the better we will become in replicating and possibly treating such intricate disorders. Like many research areas, new techniques uncover previous unknown cell types, cell states and functions, and the brain is no exception. Whether it is the recent classification of new neuronal and glial subclasses [110,111], or the classification of an entire new neuron [112], it shows the complexity that is the human brain and its functions, and the need for reliable models.

1.5.1 *In Vivo* – Human and Animal models

From the seemingly impossible task of the datasets that GWAS analysis generates, target pathways and genes are found which can be applied to more specific research systems. Most commonly, the mouse brain is used as a model system. In the mouse models, gene expression can be altered and pathways can be inhibited. Subsequently, histological and functional review of the brain and its (higher) functions can be performed as reviewed for both SZ [113] and ASD [114]. Methods such as transgenic animals, viral alteration of gene expression, pharmacological intervention of neurotransmitter function, environmental stress (during development or in adult), and electrical stimulations have been used as approaches to construct an animal model (as reviewed before [115]). Arguably, the main upside and downside is that the work is done in a rodent. On one hand, there is an actual ability to test behavioral interactions, however done in an animal that in some ways differs in cellular composition and function from humans. As recently shown, differences between mouse and human or even chimpanzee and human are highlighted. For example, the regulation of cortical neurogenesis can differ [116], human specific genes [117,118], neurons [112] and even glial function [119-121]. Despite all the differences, in the end, rodents still present a model that can lead to insights in the behavioral effects in a larger context.

1.6 *IN VITRO* - CELLULAR MODELS

Of course, the drawbacks of behavior analysis seen in animals, due to not being human, are almost insignificant compared to cells in a dish. However, with the discovery of reprogramming, a new avenue of human cells in a dish became feasible, without relying on immortalized cell lines derived from cancer patients.

1.6.1 Induced Pluripotent Stem Cells

The introduction of "reprogramming" cells to a undifferentiated state came first by Gurdon in 1962 [122], with presenting somatic cell nuclear transfer. Here, the nucleus of a somatic cell is transferred to an enucleated oocyte. In this process the somatic nucleus is "reprogrammed" by the oocyte. Many years later, in 2006, a true revolution in human cell culture was discovered. Yamanaka found first in mice, and later in human [123,124] that the introduction of four factors (Oct4, Sox2, Klf4, and c-Myc; OSKM factors) was enough to reprogram fibroblasts into pluripotent stem cells (PSCs) that resembled embryonic stem cells (ESCs). Just shortly after, Thompson's lab showed a cocktail *OCT4*, *SOX2*, *NANOG*, and *LIN28* was also sufficient to reprogram somatic cells [125]. They termed these cells induced pluripotent stem cells (iPSCs). Realizing the potential of these iPSCs, the idea of

reprogramming was awarded the Nobel Prize in 2012, shared between Gurdon and Yamanaka. Ectopic expression of the reprogramming factors was initially mediated by retroviral transduction. Like lentiviral transduction, retroviral is an integrative method, to deliver genes into the host genome, and with that the possibility for malignant transformations [126]. Since then, other, non-integrating strategies have been developed such as: Sendai viral vectors [127], proteins[128] and mRNA [129]. All have been used to induce pluripotency in somatic cells, generally using the same original OSKM factors or a combination thereof. Interestingly, Oct4 was first shown to be crucial for the reprogramming process [123,124,130]. However a recent publication showed that *Oct4* could be omitted and that the "SKM-cocktail" (Sox2, Klf-4 and c-Myc) a more faithful epigenetic reprogramming[131].

1.6.2 iPSCs to Neuroectoderm

To mimic parts of the brain, cells of the neuronal lineage are needed. Thus, the next step from iPSCs will be the differentiation towards neural ectoderm. Early protocols used embryoid bodies (EBs) followed by plating down in fibroblast growth factor (FGF)-2 containing media and isolation of neural rosette like structures resembling the neural tube [132,133] to generate some form of neural stem cell. These cells have a broad differentiation potential and the ability to be transplanted and show integration *in vivo*[132,133]. With efforts to optimize the generation of neural cells, researches had focused on signaling patterns to guide the PSCs towards neuroectoderm. It is generally considered that the pathways involving FGF, bone morphogenic proteins (BMPs) and, WNT play determining factors in patterning stem cells towards neural ectoderm [134]. BMPs play a particular important role, as it has been shown early on that disruption of this pathway leads to the induction of neuroectoderm [135,136]. And it was the blocking of this pathway that led to the generation of a protocol that creates neuroectodermal cells from PSCs with high efficiency [137]. This "dual SMAD inhibition" protocol utilized the blocking functions of Noggin and small molecule SB431542 (SB). Noggin as inhibitor for BMPs and SB as inhibitor for the Lefty/Activin/TGFß pathways, together generating a more efficient route to capture NES cells without the need of an EB stage.

1.6.3 NES cells

As stated before, NES cells form the neuroepithelium from which the brain is built. Therefore, these cells are of special interest if one wants to mimic brain development. In culture NES cells respond to FGF and EGF signaling [132,138] and have been shown to be generated from ESCs [139] and later iPSCs [140] which can

functionally integrate upon transplantation and, resemble a cell type found in the early human brain [141]. Since then, we and others have shown the usability of these cells in modeling neurodevelopmental disorders [77,142,143], increased recovery after stroke and spinal cord injury [144,145] and, certain cancers like medulloblastoma [146,147]. Neural differentiation protocols were generally developed with pure neuronal, astrocytic or oligodendrocyte purposes, and generally were achieved. However, this homogeneity can be construed as drawback for modeling the complexities of the human brain. And the practical and ethical concerns of endogenous brain tissue make post mortem, or abortion derived tissue a less reliable, although more representative model. Thus, the search for alternative models continued.

1.6.4 Brain Organoids a.k.a. "Mini Brains"

To improve recapitulation of *in vivo* neurological development, scientists started to culture neural tissue on low-adhesive plates to force the cells to self-organize, generating neural tube-like structures. These structures include a small lumen and radial orientated cells, reminiscent of radial glia that produce different neuronal subtypes [148]. Further tweaking of the protocol by excluding serum from the medium and quick aggregation, resulted in a serum-free culture of embryoid body-like aggregates-quick (SFEBq) [149]. Adding a supportive structure such as matrigel, resulted in the ability to grow even more complex whole brain organoids. These organoids consist of a lumen, lined with radial glia cells that give rise to several distinct cortical layers, and are even able to recapitulate key phenotypes of microcephaly [150,151]. Later, researchers have modified the existing protocol in order to generate less complex but more specific regional organoids (e.g. forebrain, midbrain and hypothalamic organoids) [152]. The promise of each of these techniques is that they recapitulate neuronal (disease) development and give insight into the mechanisms of cerebral development in health and disease. Relying on the self-organization of the system comes with a drawback, variability. However, further progress has been made to reduce variability [153]. Interestingly, when researches compared available datasets from different protocols, both directed and non-directed differentiation, they found a relative similarity between cell clusters albeit a slightly different path to get there [154].

1.6.5 How to get to an organoid

1.6.5.1 IPSCs

Utilizing these relative complex protocols to their full potential comes with several hurdles that scientists have to overcome. Establishing a pluripotent cell culture is laborious and demanding. Finding the right starting cells is key for the success of any protocol. Variations in pluripotent stem cell (PSC) colonies will result in inconsistent and irreproducible results. Defined cell substrates, and defined medium has been around for roughly 15 years [155-158]. This together with a standardized protocol for the generation and maintenance of PSCs will result in iPSCs that are more resistant to DNA damage [159] and will possibly yield a more homogeneous pluripotent stem cell population. One way of interpreting the homogeneity of the PSCs is the (protein) expression levels of the classical pluripotency markers OCT4, NANOG, and SOX2. These factors are the drivers of pluripotency and their expression is tightly regulated. Each factor needs to be present in a specific ratio to another in order to maintain pluripotency. Moreover, different levels are required for different lineages. For example, an increase in expression of Oct3/4 tilts the differentiation towards primitive endoderm and mesoderm [160,161]. Similar rules apply for Nanog and Sox2. Nanog represses ectoderm formation whilst Sox2 represses mesoderm specification [162]. This stresses the importance of the starting material being as homogeneous as possible regarding the expression of key transcription factors to reduce variability and increase reproducibility between experiments. Heterogeneity amongst PSCs will result in varying "levels of pluripotency", and thus discrepancies in differentiation capability. Therefore, striving for a homogeneous population of PSCs will aid in reducing variability and increases consistency between experiments.

1.6.5.2 Embryoid bodies

Most of the 3D culture methods are initiated by generating cell aggregates named EBs. There are numerous ways to generate these EBs as described by Hiroshi Kurosawa [163]. These include the use of bacterial-grade dishes, methylcellulose culture and the hanging drop technique. Although all are effective ways to generate EBs, the outcome of the EBs vary across techniques. Using bacterial-grade dishes or any other dish coated with anti-adhesive material, results in variability regarding EB size and number of EBs generated. This is highly depended on the dissociation technique used to detach the PSCs. When PSCs are allowed to remain in clumps, there is no control over amount and size of these clumps, resulting in variability amongst size of the generated EBs. As micro-patterning

techniques have shown that the size of EBs influences differentiation potential [164,165], it is preferred to generate the right size of uniform EBs to better control the outcome. The hanging drop method seems to alleviate many of the problems of other techniques. With the addition of Rho-associated protein kinase (ROCK) inhibitor that allows PSCs to be dissociated into single cells [166], scientists are able to consistently reproduce uniform sized EBs [167]. But it is laborious and ideally there needs to be a dedicated incubator to reduce the risk of falling down or merging of the drops. To date, the optimal method is to use a 96 low-adhesive round bottom well plate. These plates ensure uniformity of shape and size across EB generations. With the ability to adjust cell number per well and thus control the number of cells in a sphere, combined with easy access to change the medium, yields in an easy system with uniform results. This is necessary for reliable recognition of the stage of differentiation of the EBs.

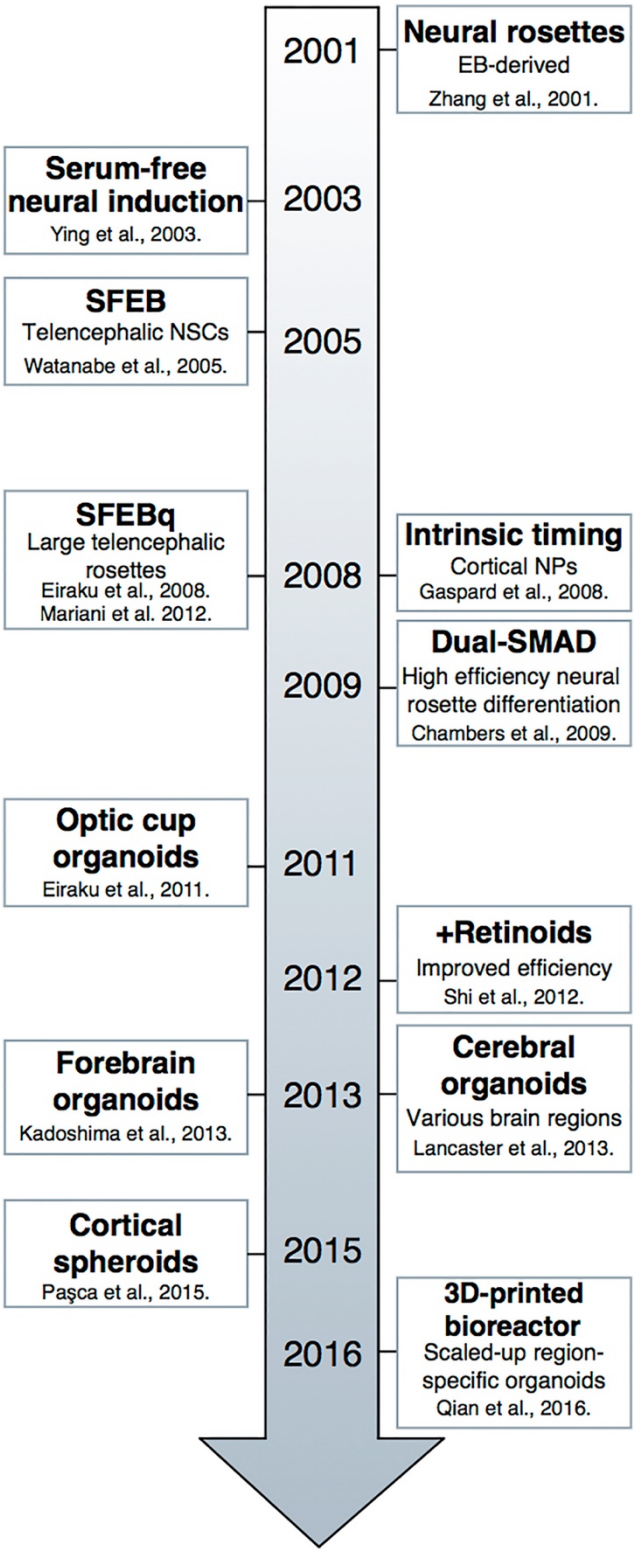

Figure 5: Timeline of Recent In Vitro Methods of Neural Differentiation. Kelava and Lancaster 2016, reproduced with permission from the journal.

1.6.5.3 Neuroectoderm

For the generation of any type of brain organoids, EBs need to be directed towards the ectodermal lineage. Luckily, when EBs are maintained in minimal medium (without serum) their "default programming" is to adopt a neural fate [168]. Due to the self-organizing capability of stem cells cultured in EB like aggregates, this represents itself as a bright ectodermal ring around a darker center. This is a crucial step before advancement to the next step in obtaining a neural fate. When EBs fail to display such a distinct morphological cue, continuation is mute since they have failed to generate ectodermal tissue. To guide differentiation further towards a neural fate, several protocols can be followed, all depended on which outcome is needed. In the last years several protocols have been developed for the generation of distinct brain regions like forebrain, midbrain, or hypothalamic [152], hippocampal/choroid plexus [169], cortical [170], cerebellar [171], whole brain organoids [150]. For a concise overview and timeline see [172] and Figure 5. Neuroectodermal tissue undergoes massive expansion during development. In order for aggregates to sustain the rapid proliferation, the brain-organoid field took lessons from work in other 3D culturing fields, most importantly, the work performed in the laboratory of Hans Clevers. There, they found that a supportive matrix is imperative to aid in expansion and self-organization of tissue *in vitro* [173]. Matrigel is a key factor in enabling the expansion of neural ectoderm.

1.6.5.4 Mature minibrains

As by now numerous publications have shown, the usability for organoids is seemingly endless. The identification of functionality in the organoids and the resemblance they have to human brain development, becomes more and more apparent. By combining dorsal and ventral organoids, researchers have created a model in which they could trace the ventral neurons migrating to their dorsal destinations [174,175]. Not only the migration between different brain regions is essential for brain development, but also the integration and feasibility to form functional neuronal networks is the key to a functioning brain. It has been shown that organoids form light sensitive photoreceptors that alter their electrical activity upon light stimulation. This is indicative not only for the generation of inter organoid connectivity, but also for a functioning network [176], and therefore the possibilities to test connectivity functions. To further the field, some limitations needs to be addressed. Generally relying on self-organization without (too much) inhibitors to guide organoid formation, results in variability. It has been shown that different protocols result in similar cell types generated, however with distinct preference in differentiation pathways to get there [154]. Furthermore, the lack of

generating all six cortical layers and the disappearing of the distinct progenitor layer over time are crucial factors to address [172]. Possibly the lack of a vasculature system, depriving the inner cells of nutrients, is a fundamental step to overcome. Thus far that is on its way to being solved by co culture and transplantation to the mouse brain [177-179]. It is needed to slightly alter the different protocols to generate the diversity of cells types of the brain. Researches have generated microglia [180], astrocytes [181-183] and, oligodendrocytes [184,185] inside organoids and it seems just a matter of time before the protocols are combined and resulting in the generation of more of the brains' cell types in a proper timeframe.

2 AIMS

With this thesis the aim was to generate and use brain development models, find the limiting factors, and overcome them. To achieve this, we have utilized a combination of approaches ranging from 2D to 3D models.

Roles each paper fulfilled:

- ***Paper 1 – An in vitro model of lissencephaly: expanding the role of DCX during neurogenesis***

  Using a classical neurodevelopmental disorder (lissencephaly) to test the feasibility of 2D NES cells to replicate certain aspects of this disorder.

- ***Paper 2 – p53 controls genomic stability and temporal differentiation of human neural stem cells and affects neural organization in human brain organoids***

  Combining the 2D NES cells and 3D organoids to affirm the role of p53 in neurodevelopment and to explore a phenotype that does not translate from mouse to human.

- ***Manuscript – Generation of a 2D and 3D System to Understand Neuropsychiatric Disorders***

  Taking advantage of the *in vivo* likeness of 3D organoids and translating them to a format that would allow for efficent 2D screening possibilities, as well as 3D applications.

3 RESULTS AND DISCUSSION

3.1 PAPER I – AN *IN VITRO* MODEL OF LISSENCEPHALY: EXPANDING THE ROLE OF DCX DURING NEUROGENESIS

In this project we sought to generate a model system for what has been described as a typical human disorder, lissencephaly, also known as "Smooth brain disease". When caused by mutations in DCX, smooth brain disease is characterized by the disorganized lamination of the neocortex in males and subcortical band heterotopia in females, as it is located on the X-chromosome [57,58]. The disorganization of the cortical layers is not recapitulated in mice [64], and thus, with our access to patient material and ability to derive neural stem cells from these patients, we explored the feasibility of an *in vitro* model for lissencephaly.

Lissencephaly is a disorder with a neural migration phenotype. Neural migration is plainly movement of a neural cell from one place to another, usually attracted and repulsed by chemo- attractants and -repellants signaling on a growth cone like structure situated on the end of a neurite [186,187]. We have shown the reflection of this principle in our lissencephaly model system. We show that in patients with a DCX mutation the migration of newborn neurons is impaired and that the neurites of these neurons are shorter. Interestingly, we could rescue the shorter neurites not only by the KD of the chemorepellent *SLIT3*, but as well by adding the SLIT3 ligands ROBO1/2. When researchers generated iPSCs from lissencephaly patients harboring missense mutations in *TUBA1A,* coding for a tubulin protein present in neurons, they also found decreased neurite length [188]. This shows the reproducibility of this phenotype in the same disorder with a different causative mutation across laboratories.

During brain development, neural stem cells need to ultimately generate post-mitotic neurons that migrate away from the stem cell niche and establish themselves in the correct place and integrate into the network of axons and dendrites. In our DCX model we lastly showed the prolonged proliferation of neural progenitor cells using a neural differentiation protocol. Again, this can be viewed as one of the classical hallmarks of lissencephaly, in which the cortex is significantly thicker [57,189]. Presumably, due to the prolonged proliferation period we found and increased number of proliferation cells in the DCX patients. Recent analysis of our transcriptome data does reveal an overrepresentation of *FABP7* and *HES5*. *FABP7 (BLBP)* a radial glia marker in the CNS [29] and *HES5* involved in the neurogenic to gliogenic switch [190]. Both indicate an interesting line of investication that can be

followed up on. Either a more radial glia like profile or the generation of astrocytes that happens in the differentiation of DCX patient cells can be a new phenotype in DCX-lissencephaly patients.

3.2 PAPER II – P53 CONTROLS GENOMIC STABILITY AND TEMPORAL DIFFERENTIATION OF HUMAN NEURAL STEM CELLS AND AFFECTS NEURAL ORGANIZATION IN HUMAN BRAIN ORGANOIDS

Here we sought to establish a human *in vitro* model for brain development involving one specific gene: *TP53*. Uncovering the role of p53 in human brain development we came across a plethora of genes. A total of 2159 genes are deregulated in *p53*KD NES cells. We used cell lines derived from two healthy individuals, and KD p53 with the use of two different shRNAs targeting different parts of the p53 transcript. We confirmed the "guardian of the genome" to be safeguarding chromosomal rearrangements and to be an inducer of apoptosis upon DNA damage in our neural stem cells. Interestingly the loss of p53 did not result in cells exiting the stem cell state, but rather primed the cells for differentiation. Surprisingly, upon the KD of p53 in human NES cells, we observed a slower proliferation rate of these neural stem cells. This is in contrast to observations from isolated cells from the *p53* KO mouse brain [191-193], where more proliferation as measured by neurosphere assay, is observed. This might be due to several factors and it would be of great interest to study where the discrepancies arise from. One factor could be species-dependent. It has been shown that human and mouse only share 44% of the gene regulatory network. Moreover, 1000 genes in the p53 network are differently regulated [194,195]. Another variable to consider would be the developmental stage of the cells. In our study we used NES cells to show a reduced proliferation rate. NES cells are a cell type that represent a population of neural stem cells in the human brain that is present around gestational week 5-7 [141]. Several studies reporting enhanced proliferation used adult mouse SVZ [192,193], or cells isolated from the olfactory bulb [196]. Contradictory, researches have isolated NPCs from E13,5 *p53* KO mouse brains, and did not detect increased proliferation, or after a BrdU pulse in the E16 telencephalon [197]. In summary, all research performed so far yielded different outcomes. This stresses the fact that not only the method but also the timing and the location in the brain are important variables to consider.

It is not all different between mouse and human neurogenesis. The general notion of p53 safeguarding the stem cell state and that loss of p53 results in differentiation, is a phenotype that is partially recapitulated in the human NES cells. Although the NES cells do not leave their stem cell state upon loss of p53, when

they are allowed to differentiate by removal of growth factors, they form more mature neurons faster. This accelerated differentiation is also seen after radiation. The *p53* KO dentate gyrus reveals an increased generation of neurons [198], as well as increased proportion of differentiation markers in isolated neurospheres [191,197]. Furthermore, spanning this phenotype across cell types, the mesenchymal differentiation of *p53* KO cells was only half the time of WT *p53* cells [199]. This indicates that the regulation of differentiation by p53 is conserved across species and observed in different cell types and systems.

Across species, the regulation of certain metabolic processes also seems to be conserved. However, as reviewed previously [200], whether p53 promotes or inhibits OXPHOS, glycolysis and/or, the TCA cycle could be tissue dependent. As our results show, metabolism is one of the major affected pathways in the *p53* KD NES cells. Downregulation of the OXPHOS cluster genes, verified by the downregulating of DECR1, resulted in a glycolytic shift with upregulation of *HK2* in our NES cells. Furthermore, PSA-NCAM$^+$ isolated cells from our 3D organoids showed a similar downregulation of OXPHOS genes phenotype. Therefore, it is clear that p53 is a key regulator in balancing the cells' energy needs by regulation of the metabolic pathways.

To reflect on *in vitro* neurogenesis in greater context, we used organoids to mimic brain development. Using a system that has a high degree of transcriptional and epigenetic similarities to human brain development [201,202], we showed the effect that KD of *p53* has on brain development. Most strikingly, the disorganization in cortical formation. SOX2$^+$ stem cells are found outside of supposedly neurogenic regions, accompanied by its supposed daughter cells, TUJ1$^+$ neurons. Interestingly, disorganization of cortical regions was reported in *53BP1* (encoding a p53 binding partner) KO organoids [116]. Further quantification of the stem cell niche revealed a decrease in both TBR2$^+$ progenitors and TBR1$^+$ neurons. With the conformation that p53 is still very much downregulated, it is unquestioned that this has a major role in the observed phenotype. Continued analysis of the stem cell niche, showed at first counterintuitive results. The expression of cell cycle marker Ki67 was unaffected. At the same time, we could not observe differences in Ccas3$^+$ cells, which at this timepoint would possibly indicate that the defect in neurogenesis resulted from an earlier phenotype. However, when analyzing the cell cycle with EdU, we observed an accumulation of cells in G1phase at the expense of S-phase indicating a slower proliferation rate, quite possibly a contributing factor to the diminished progenitor and neural population.

Interestingly, metabolic regulation is shown to be key for neurogenesis. Not only in *in vitro* neurogenesis [95,203], but also in hippocampal neurogenesis [102]. Inhibition of mitochondrial complex function results in a suppression of neurogenesis, specifically at the IPC level. in our two systems, *p53* KD NES cells have reduced mitochondrial complex function, and isolated *p53* KD PSA-NCAM$^+$ cells show a metabolic gene imbalance towards decreased OXPHOS. In summary, we hypothesized that in humans, p53 regulates neurogenesis, possibly through regulating energy pathways.

3.3 MANUSCRIPT – GENERATION OF A 2D AND A 3D SYSTEM TO UNDERSTAND NEUROPSYCHIATRIC DISORDERS

In this manuscript we sought to combine the best aspects from both paper I and paper II and apply it to neuropsychiatric disorders.

We show that the generated stem cell layer in the organoids resemble that of a mouse brain. The generated TBR1$^+$ cortical layer is clearly separated from the stem cell layer and some RELN$^+$ cells made their way to the upper layers of the cortex. Further on in differentiation, GLAST$^+$ radial glia appear together with sporadic GFAP$^+$ fibers. Which glial population these fibers represent needs to be determined. The presence of GFAP in the developing brain can be attributed to either astrocytes or radial glia, with the delta isoform of GFAP (GFAPΔ) lines specifically the human ventricular zone [27]. This specificity is not appreciated in the mouse brain, where GFAPΔ is more widely spread between astrocytes, neurogenic, and non-neurogenic stem cells [28]. We further show the ability to generate organoids from an ASD patient and SZ patient. These organoids show hints of developing the oSVZ layer with HOPX$^+$ oRG cells. oRG cells are thought to be a major contributing factor to the expansion of the human neocortex [40], and therefore of vital importance to model human brain development.

Part of what makes us human, is how information is processed. In the brain that is done via neurons and their action potentials. Interestingly, when comparing macaque to human neurons in the amygdala and the cingulate cortex, the human neurons were more efficient with a higher information capacity (spikes are more efficiently distributed) [204]. The authors postulate a correlation between the tradeoff seen with a higher efficiency and lower robustness to a possible propensity of human psychopathologies. In the developing human cortical plate, some neurons that are capable of firing a single provoked action potential (AP) are present as early as 16 weeks into the gestational period, but repeated APs were not present

before week 20 [205]. This falls relatively well in the timeframe described of recordings from organoids where no spontaneous APs were detected after four months of differentiation (~16 weeks), but ample activity was visible after eight months (~32 weeks) [176]. Further comparison of organoids development to network activity from neonatal fetuses showed remarkable similarities [206]. As the authors state, there are many factors to take into consideration. Interreference of the skull, electroencephalogram (EEG) acquisition and electrode placement are all cofounding factors. Similar to *in vivo* recordings, the *in vitro* data is variable. Starting from single cells could yield slight population differences in early stages that will have profound effects later on the differentiation. In addition, placement on the Multi Electrode Array (MEA) and organoid manipulation are confounding factors to signal acquisition. Taking this into account, the observed similarities are striking. There is significant correlation of organoids from 25 weeks and on to the developing human brains' network formation. Over the course of differentiation both burst frequency and synchronization kept on climbing from two to the tenth months. While these correlations were not observed in mouse derived primary cells and 2D monolayer iPSCs.

Since the emergence of electrical activity has a relative long timescale, we explored an alternate way to assess neural network dynamics. Calcium network dynamics have been used previously to assess functionality and maturity of neural networks [142,207,208]. Our subset isolation confirmed the increased cell specificity obtained after magnetic activated cell separation (MACS), and allowed for plating and subsequent calcium dynamic analysis. This showed a gradual maturation over time as indicated by the shorter spike intervals and increased amplitude of the transients, as observed previously in maturing cultured neurons [209]. Therefore, we show that the approach of determining maturation and network formation from an isolated neural population works, and is in line with previous findings. However as discussed previously, 2D neural networks have a relatively poor correlation with the developing human brain [206]. Therefore, in depth studies might be better suited to be tested in an organoid like system. However, because ease of use and inexpensive costs, initial drug screening for effectiveness or toxicity would be still a suitable application for 2D derived neural systems.

To combine the usability of a 2D system with the apparent *in vivo* similarities of the organoids we analyzed the survival and differentiation of transplanted NES cells in neuroectodermal like structures. GFP expressing NES cells were injected into the organoid and allowed to differentiate for 30 days. Not only did the cells survive the

transplantation, they also differentiated to some extent. As previously demonstrated, NES cells mainly form GABAergic neurons upon differentiation [140]. This is also apparent in our transplanted cells. Large GFP^+ clusters surrounding $GABA^+$ cells were apparent, with possible some $FOXG1^+$ cells intermingled. If confirmed to be true, it would indicate some degree of intrinsic flexibility in stem cell fate or retained heterogeneity in the stem cell population. It has recently been shown that NES cells are heterogeneous to some degree, and that in the stem cell state populations with the potential of generating excitatory, inhibitory, and gliogenic cells exist [210].

With this work we show the usability and feasibility of organoids as a model system, as well as some limitations that can be taken into consideration to study neurodevelopmental disorders.

4 CONCLUSION AND FUTURE PERSPECTIVES

Gaining greater understanding in pathways involved in human neurogenesis is essential to understand the workings of our brain. But what the functional outcomes are is equally important however, often much more difficult to obtain. Our example, human brain development, is for obvious reasons difficult to study in humans. Obtaining human material is often variable and cumbersome therefore we aim to increase the understanding and usability of *in vitro* systems. The focus on *in vitro* does come with considerations and the different aspects of a simpler, more homogenic 2D neural stem cell system all the way to whole brain organoids that offer a wider variety of cell types and interactions (Figure 6).

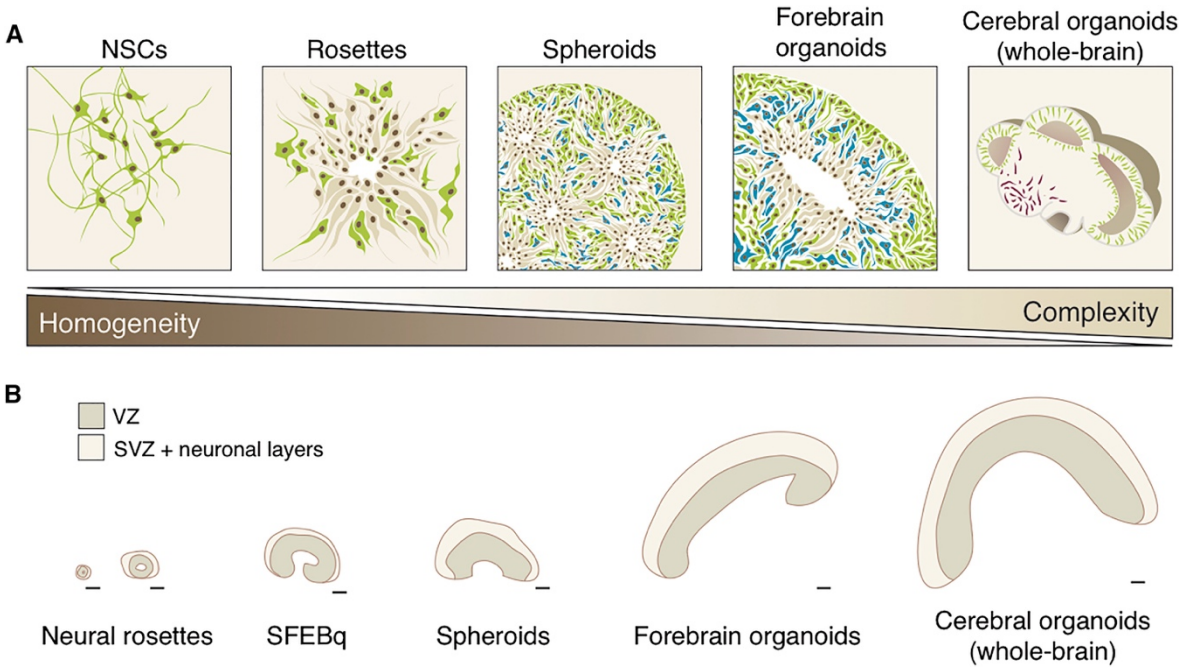

Figure 6: The Trade-Off between Homogeneity and Complexity. Keleva and Lancaster 2016 (A) A scale showing the relationship of the complexity of the cells/tissue produced by individual protocols and the homogeneity of the cells/tissues generated. For individual methods, please see main text. The individual cells/tissues are not shown to scale. The color scheme does not represent the exact number of cell types. (B) Cartoons depicting individual regions of cortical tissue from various methods demonstrating their relative sizes. Traces were performed on actual images from the following studies in left to right order: Neural rosettes: Figure 3d-i from Chambers et al. (2009) and Figure 1d from Kirwan et al. (2015); SFEBq: Figure 6N of Eiraku et al. (2008); cortical spheroids: Figure 2a from Pasxca et al. (2015); forebrain organoids: Figure 3A from Kadoshima et al. (2013); cerebral organoids: Figure 4 from Karus et al. (2014) (this is an image of a cerebral organoid cultured as in the original Lancaster et al., 2013). Images chosen for the tracing were at similar stages of development based on timing and thickness of the VZ compared to outer regions. For those with multiple matched images, the largest was used for tracing. All images are scaled to one another; scale bar in all images represents 100 mm. Reproduced with permission from the publisher.

With the realization that more and more aspects of the human brain are unique to homo sapiens such as progenitor cell specific NOTCH signaling in cortical development [211], or the duplication of a gene, that resulted in *ARHGAP11B*, that when introduced in the mouse possibly promotes gyrification [118], one of the defining factors for the increased cortical area seen in humans. Notably, the localization of this human specific protein has recently been shown to be in the mitochondria of basal progenitor cells, where it promotes proliferation trough glutaminolysis [117].

Further work also needs to focus on astrocytes, since they might outnumber neurons in the brain [212], but are distinctly more complex and on average 2,6 times larger with ten times more processes than their mouse counterparts [120,121]. Interestingly, this realization has created a new niche of investigation, namely human glial chimeric mice. Although it is not a new notion to generate chimeric rodents with the transplantation of human precursor cells [213], the realization of the complexity of the human brain has made researches searching for increased human likeness in their models. Mice transplanted with glial precursor cells showed an increased enhanced learning ability and plasticity of the host [119], and are continuing to show their involvement in neuropsychiatric disorders such as schizophrenia [214]. Therefore, integrating human cells in research would be a step to consider to improve translation to patients.

Lessons from the previous two papers and manuscript brought this thesis to its purpose. The question lies before us on what would be a suitable approach to study neurodevelopmental disorders. What we have generated here, attempts to tie together form and function. Form, in the way that histologically, brain development is recapitulated. Development of the different cell types that make up the brain and their proper placement, is of course, the cornerstone of any developmental project. As we have seen in **paper I**, migration defects and, neurite projection are major phenotypes in lissencephaly that we could recapitulate *in vitro*. The model system did however lack ability to mimic the organizational structure found in the brain, as is true for many 2D neuronal systems. It did allow for the recapitulation of known *in vivo* phenotypes such as migration defects

At the start of my PhD I remember distinctly saying that I do not want to work on cancer, and here I am, coming dangerously close. It is true that any given protein on its own does not equal cancer, but as far as cancer associated proteins go, p53 is at the top of the list. Not surprisingly though, it is the most altered gene in human

cancers [215]. Depending on who you ask, it is either the most important gene in life or, "it is that gene that the cancer people play with". However, **Paper II**, made me realize the similarities. On a basal level, the glycolytic phenotypes in cancer and the seemingly similar profile of neural stem cells in which *p53* is knocked down made way for the field of (cancer) metabolism to guide us in understanding the phenotypes seen in our 2D and 3D cell models. This paper was challenging and interesting in many ways. Most intriguing the seemingly conflicting results of enhanced or accelerated neural differentiation from our NES cells, and the observed impairment of neurogenesis in our 3D organoids. I think it is important to mention this, and without trying both systems side by side we possibly would not have seen both phenotypes. Comparing these systems is of course unfair in many ways. The NES cells had a "normal", albeit 2D development towards neuroectoderm before KD of *p53*, whereas the organoids dealt with KD of *p53* from the iPSC stage. Opening up the possibility to altered mechanisms during initial differentiation towards neuroectodermal fate.

Learning from paper I and paper II, in the **Manuscript** we sought to combine the strengths from both 2D and 3D cultures, and applied them to neuropsychiatric disorders. Here the greatest challenge was to find ways to combine functionality assays to cell culture that could resemble human brain function. Fortunately, there are lessons to learn from the mouse and the study of its brain. Since the focus was neuropsychiatric disorders, we aimed for neuronal functionality, the most common drug target. Interestingly, when researchers compared preterm EEG with organoids, 2D control iPSCs, fetal cells and mouse primary culture, the correlation of 2D and mouse cells was actually negative. Meanwhile, in ascending order of Pearson correlation, 2D fetal cells (0,1), organoids (<25 weeks, 0,4), organoids (>25 weeks, 0,6), and lastly, held out neonatal recordings (0,75) showed significant similarities with the developing brain [206]. This together with several reports on the transcriptomic similarities of organoids to human brain development [153,154,176,201,202,206,216,217], does make a strong stand on the usability and resemblance of brain organoids to fetal brains. However recently a possible cell culture effect has been explored on 3D cell culture. When organoids and isolated fetal brain tissue was grown in cell culture researchers saw markers of endoplasmatic reticulum stress upregulated [218,219]. And not only in tissue, in their starting iPSCs these markers were present. Interestingly, when cells from the organoid were transplanted to the mouse brain, these markers seemed to be downregulated again.

Together the work in this thesis shows that *in vitro* cell culture can be a relative good model to address pathologies that animal models lack. However, like all models, we need to take into consideration the limitations of *in vitro* systems like 2D neural stem cells or 3D organoids. The lack of cell types able to interact with each other, nutrient flow, and mitogens from different parts of the body are just a few examples of factors we have yet to control *in vitro*. Therefore, I would like to close this thesis with my opening statement:

"Essentially, all models are wrong, but some are useful"

George E.P. Box

5 ACKNOWLEDGEMENTS

"It takes a village" is the most applicable phrase I can think of that describes the journey that resulted in this thesis. Without the guidance, challenge and laughter from all of you I would not be where I am today, therefore I'd like to thank the people that gave me what was needed throughout this process.

My principal supervisor **Anna Falk**, thank you for allowing me to peruse my own ideas, without that freedom this theses would not be what it is today.

My co-supervisor **Klas Blomgren**, unfortunately we have not worked together intensively but perhaps that will change in the future.

Thank you, **Eva Hedlund**, my co-supervisor. The meetings with you and your lab gave me the perspective I needed. You are a caring, brilliant scientist that always can make time to put life in perspective. For me you have been an inspiration and I wish you all the best in work and in life.

Margareta Wilhem, collaborator or unofficial co-supervisor, the line is a bit blurry. But I do know, I value your dedication to science being done properly. Thank you for all you help

Marie Arsenian Henriksson, I consider you one of my unofficial supervisors and I want to thank you as well for all the help you have given over the last years. The support, the checking if everything is going ok, and the constant search for ways to help are really appreciated and sometimes undervalued. All those little things meant a lot to me. Thank you.

I want to thank my collaborators **Fred Gage, Julian Walfridsson, Per Uhlén, Thomas Helleday, Ali Mirazimi, Niklas Dahl** and their labs, thanks to you for expanding my view of research and the amazing things I could be involved in

And a thank you for my previous PI's and supervisors, without you I would never come this far in the first place. **Ingrid Strömberg** and **Ana Virel** up in Umeå thank you for one of the first encounters with neuroscience. **Elly Hol** and **Carlyn Mamber**, thank you for introducing me to astrocytes. **Carlyn** the amount of dedication and respect for animals you have is something I'll remember the most. Thank you for so many great lab lessons and if I remember correctly the NIN-beer pong. All the best in the future. **Vivi Heine**, **Stephanie Dooves** and **Prisca Leferink**. Dank jullie wel

voor de gezellige omgeving die jullie hebben gecreëerd, ik heb veel terug gedacht aan mijn tijd bij jullie en de technieken die ik bij jullie heb geleerd hebben me veel geholpen met de uitdagingen tijdens mijn PhD. I've had a blast with **Stephen** and **Dwayne** and I wish all of you the best in the future.

Members of the Falk lab and iPS core. Generally all of you for listening and giving input wherever you could. **Harriet** you have a great taste in books, even though you wait for the Swedish translation ;) thank you for just being you, the directness you possess is a welcome change in everyday life in Sweden, **Kelly** we cannot, not mention your baking, thank you for trying to tie us together through lemon cake, and not to mention the lifesaver you are in times of need and power outages, **Lingjie** and **Tingting** good luck with the cells, **Mastoureh** for initially training me in the lab and introducing me to NES cells, **Malin, Matti**, **Mohsen, Elias** all the best in life. And for the hours and hours and hours in cell culture, from Michael Jackson Fridays to the cooked lunches for us, thank you **Salma**.

My main (explosive) rock and dear friend **Ana**. The situations we have been here are too many to describe, but almost all of them involve: too much cell culture, too much fun or too much for life in general. I'm so happy I could share the bench with you, I can't imagine what would have become of me if we were not collages. Thank you for being you in general, the energy, the laughter, the discovering of new places half way across the world or some random bar just around the corner.

Philipp, my student. I've probably (unintentionally) put you through more than is expected from a student but you handled it all methodically and I can't express enough how much I admire the way you continued the work when I was not able, and I'm sure you do great in the future.
Astrid officieel niet mijn student maar ik neem toch wel een beetje verantwoordelijkheid voor jou tijd hier. Dankjewel voor de hoeveelheid werk die je hebt geleverd voor het project, je bent briljant en ik weet zeker dat je het goed gaat doen/doet in NL.

Than a thank you for my quarter members. First **Ola Hermanson** and his lab. **Kuba,** you guys always made the lab more cozy, the music and the disco ball are a great addition. I still think we win the prize for best organized Christmas party, although I'm slightly biased. **Julio**, **Nigel**, **Christoph** and **Jik**, Thank you for adopting me in lab meetings and all the input you gave. The people of the **Carmine Belin**, **Ceccatelli**, **Chrast**, **Harkany**, **Hökfelt**, **Ibanez**, **Kiehn**, **Lallemend** and **Olson** labs. Especially since our move to Biomedicum, the presence of each of you has contributed a nice quarter, all the random little conversations in the corridors or at the coffee machine make it that much nicer. **Joanne**, bedankt voor het antwoorden van mijn random vragen het is altijd leuk, en soms nodig, om even op z'n Nederlands te zeuren. **Frederik**, a great example why I like the Danish (the ones I've met). Funny and caring, I hope you can stay out of the wet-lab. All the best in you PhD, and life after, and maybe lift some more weights ;) **Lua**, best of luck, don't let the cells get the better of you. **Carmen**, best of luck in the PhD and now balancing with the little boy, whether it was at parties or around the lab it was always nice to have little chats with you. **Ana Olivera**, you've only been in our quarter relatively short but it was a nice addition to make our little corner more friendly. 3th time is the charm ;), good luck with everything.

In the department: **Maya** always nice to meetup, generally at a random party or the neuro fika's. **Cassandre**, you're a fun person, all the best in you PhD. **Ayoze**, Thank you for the fun Christmas party and little coffee breaks. Looking forward for some more fun and beers, probably Fridays ;)

Thank you the Zika team, **Marjo**, **Marianna** and **Aleksandra**, it was always great to meet you guys, such an easy collaboration and I wish you the best of luck.

CAM, visiting only briefly time but I thank you so much for the time spent. You were exactly what was needed to make that summer a bit better in cell culture, thank you for all the help in eradicating the unwanted incubator occupants and the lifesaving move to SciLife during our move. It was the slowest most precious walk I ever had to do in my life and I thank you for arranging the makeshift portable incubator.

My secondary department, **MTC,** and the great people I've met at the cancer side of life. Thank you **G** for being there when needed, for never stopping to talk, for my little visit to KS, for the endless support and so much more. You are an amazing caring person whom without this journey would have been much more difficult. **Maria V**, **Lourdes**, **Aida**, **Tong**, thank you for the help with random questions and for making me feel more at home in MTC. **Jojo**, always fun to play squash, good luck in finishing up, and graduating. **Jonathan** thank you for being my chair during the defense but more importantly, I thank you for the beers and parties. **Amanda**, now all the way over in UK (I hope they let out of there at some point ;)) thank you for the MTC pubs, tequila, Slakthuset, and Dovas nights. **Adam**, as much an MTC-er as me, and usually we've met in the animal house, but thank you for being just a fun person with an incredible amount of perseverance.

Thank you all the people who have help me keep my (relative) sanity. The most stable constant in recent years the Wednesday **BP crew**. First of all thank you **Phil** and **Sara** for educating me in the new beer of the month, and letting us treat BP a little bit like home. **Eliane** de geweldige Honduran/Nederlandse in Stockholm, naast je dedicatie voor je werk waardeer ik je vriendschap enorm. Je bent er altijd, of het nu voor een praatje of een biertje (2, want op 1 been kun je niet lopen) is. Heel veel succes met het afronden en tot woensdag ;). **Vilma** all those years ago we tried, and failed, a lot of experiments, but we are way better at being friends than colleagues (although I fully blame the cells). I can't wait for the next beer, or wine with a water for you, and just going over the past week, truly one of the people that have made Stockholm home for me. **Elin,** my Swedish alter ego, thank you for being amazing. Whether it is Barcelona, a Spanish mountain village, a random Swedish archipelago island, or Cologne, you are always fun to hang out with, thank you for your care and friendship. **Michael**, another awesome Dane. Thank you for endless listing and great advice to put things in perspective. You are the friend we all need, life of the party but making sure we all get home. **Konstantinos** our yogi, and probably master of the high-five, thank you for always bringing some joy to the conversation. **David**, sweet gentle giant. Thank you for always being optimistic, it's needed at times, and it has helped me more than I can say. **Kim**, thank you for all the puns, it is an amazing skill. **Aga**, always kind and hope to see you soon, **Andre**, it was great meeting you, all the best. **Christoph** and **Gert-Jan**, thanks for answering my ignorant sequencing questions and then not holding it against me that I never got to the point to actually use it. **Daniëlle**, altijd recht door zee, mede door jou heb ben ik de Nederlandse trekjes meer gaan waarderen. **Enrico**, you always bring some life to our group. **Johan** thank for accepting us weird scientists,

Sijme and **Sophie**, in irregular intervals you can make it to our Wednesdays but it's always great fun to catchup.

Giuseppe, you're dedicated scientist and it was great to meet you, I wish it'll be an smoother ride for you to the finish. **Christina** thank you for agreeing with me that sometimes things are just not nice or fun or happy. **Alex** the Mediterranean whirlwind I was in no way prepared for. Thank you for always caring and trying to help. **Petra** my fellow organoids enthusiast, thank you for those early beginnings, trying to figure out how they work.

From basics to clinics people, thank you for both organizing and participating in one of KI's best conferences. **Simona**, I think winter conference record holder and together with **Katrin**, I've learned recently great karaoke performers. Thank you for joining in the fun times.

Dalton mensen **Beuk**, **RR**, **Tessa**, **Gitta**, **Janine**. Hoelang is het alweer dat we elkaar kennen, Beuk zou zeggen, veel te lang. Git het is verschrikkelijk dat je hier niet bij kunt zijn. Je hebt geen enkele van m'n diploma's gemist en ook deze laat je niet voorbij gaan, we denken aan je zodat je er toch bij bent. Beuk, RR, Tes, Janine, since de VMBO en HAVO, hoogtepunten of dieptepunten ik waardeer enorm dat jullie er zijn en nu ik klaar ben met al deze ongein lukt het vast om elkaar vaker te zien dan eens in de 2,3,4,5 jaar.

Laura, **Jip**, **Bas**, **Derek**, **Fiona**, **Marcel**, **Adrie**, **Bianca**, **Danielle**, **Maurice**, **Ralf**, **Roelien**, **Kelly**, **Bastiaan**. Lang geleden in een klein stadje genaamd Leiden hielpen jullie me met trips naar London, Hongarije en Umeå een en al gezelligheid te brengen in het studenten leven. Dank jullie wel en veel succes in de toekomst.

NIN people, **Jackelien**, **Sybren**, **Amu**, **Lizz**, **Inger** en **Jurrien**, het was altijd gezellig in onze NIN-bibliotheek of het nu vragen waren over graphpad, experimenten, cookie Friday, of de borrels die we ook na het NIN hadden, door jullie was mijn tijd in het NIN een memorabele.

Als laatste, mijn familie, ik heb het niet makkelijk gemaakt om naar Stockholm te verhuizen maar jullie hebben het wel makkelijker voor mij gemaakt elke keer als ik terug vloog door gewoon de simpelste dingen, van gebakken vis met **Oma** en **Opa** Kerst met iedereen, **Marielle**, **Johan, Hans**, **Karin** en **Britt**, **Lot** (tut), **Fleur**, **Papa**, **Els**, **Robert** en de neefjes **Mick** en **Seth** ik hoop dat jullie dit ooit lezen en dat het wat duidelijker is wat ome Coco allemaal heeft uitgespookt in het verre Zweden.

Moeders en **Zus**, dank jullie wel voor het geloof en de support, dan wel niet op afstand. Jullie zijn de sterkste personen die ik ken en met jullie kunnen we alles aan. We have the power, honderdduizendkussen.

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